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Differentiation within the genus *Leptocarabus* (excl. *L. kurilensis*) in the Japanese Islands as deduced from mitochondrial ND5 gene sequences (Coleoptera, Carabidae)

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The phylogenetic trees have been constructed for the mitochondrial ND5 gene sequences from the Japanese *Leptocarabus* ground beetles, which contain 101 specimens collected from nearly the complete distribution ranges of them consisting of five morphological species, i.e., *Leptocarabus procerulus*, *L. kumagaii*, *L. hiurai*, *L. kyushuensis* and *L. arboreus*. On the trees, there are recognized two major lineages, each of which is further divided into two or more sublineages. The phylogenetic lines are geographically linked. Two or more species occur in a single lineage, and the same species appear in different lines. We suggest that transformation from one type of morphology to another took place in parallel in various periods of evolution of the Japanese *Leptocarabus*. From the phylogenetic tree and the dating from the nucleotide substitution rate and the geohistorical data it is inferred that the ancestry of all the Japanese *Leptocarabus* species was derived from a protoform *of L. kyushuensis* inhabited the ancient Japan area, followed by separation into two lineages after split of the Japanese Islands from the Eurasian Continent. They then propagated distribution to occupy their own habitat ranges, during which the morphological transformation took place in some lineages.

INTRODUCTION

The Japanese *Leptocarabus* species have been well studied taxonomically and are classified into the following species: *L.* (s. str.) *procerulus*, *L.* (s. str.) *kumagaii*, *L.* (s. str.) *hiurai*, *L.* (s. str.) *kyushuensis*, *and L.* (*Adelocarabus*) *arboreus* (Nakane, 1962; Ishikawa, l991). Each species, except *L. kumagaii* and *L. hiurai*, is further separated into a few, or sometimes many, subspecies (local races). Indeed, *L. arboreus* is divided into 20 subspecies based on minor morphological differences (Ishikawa, l992). A distribution map of these species is shown in Fig. 1 after Tominaga and Hiura (l979), where the subspecific rank is not considered except for some, because of no influence on discussion of this paper. Despite the detailed taxonomic studies, little had been known on the origin of, and the phylogenetic relationships among, the Japanese *Leptocarabus* species and subspecies until the phylogenetic trees of mitochondrial NADH dehydrogenase subunit 5 (ND5) gene and of 28S rDNA from the Japanese and the continental *Leptocarabus* species were constructed (Kim et al., 2000). The main conclusions reached by Kim et al. are that there has been found no direct ancestry of the Japanese *Leptocarabus* species in the Eurasian Continent, and the ancestor of the Japanese species would have inhabited in the ancient Japan area of the continent, followed by its diversification into several species after separation of the Japanese Islands from the continent. Kim et al. also presented a phylogenetic tree of the ND5 gene from the Japanese *Leptocarabus* species that are separated into two lineages, one containing *L. kyushuensis* and another including all the rest of species. Since no detailed discussion has been made by Kim et al. (2000), the phylogenetic relationships among the Japanese *Leptocarabus* species still remain largely ambiguous, and the detailed account on this problem is presented in this paper.

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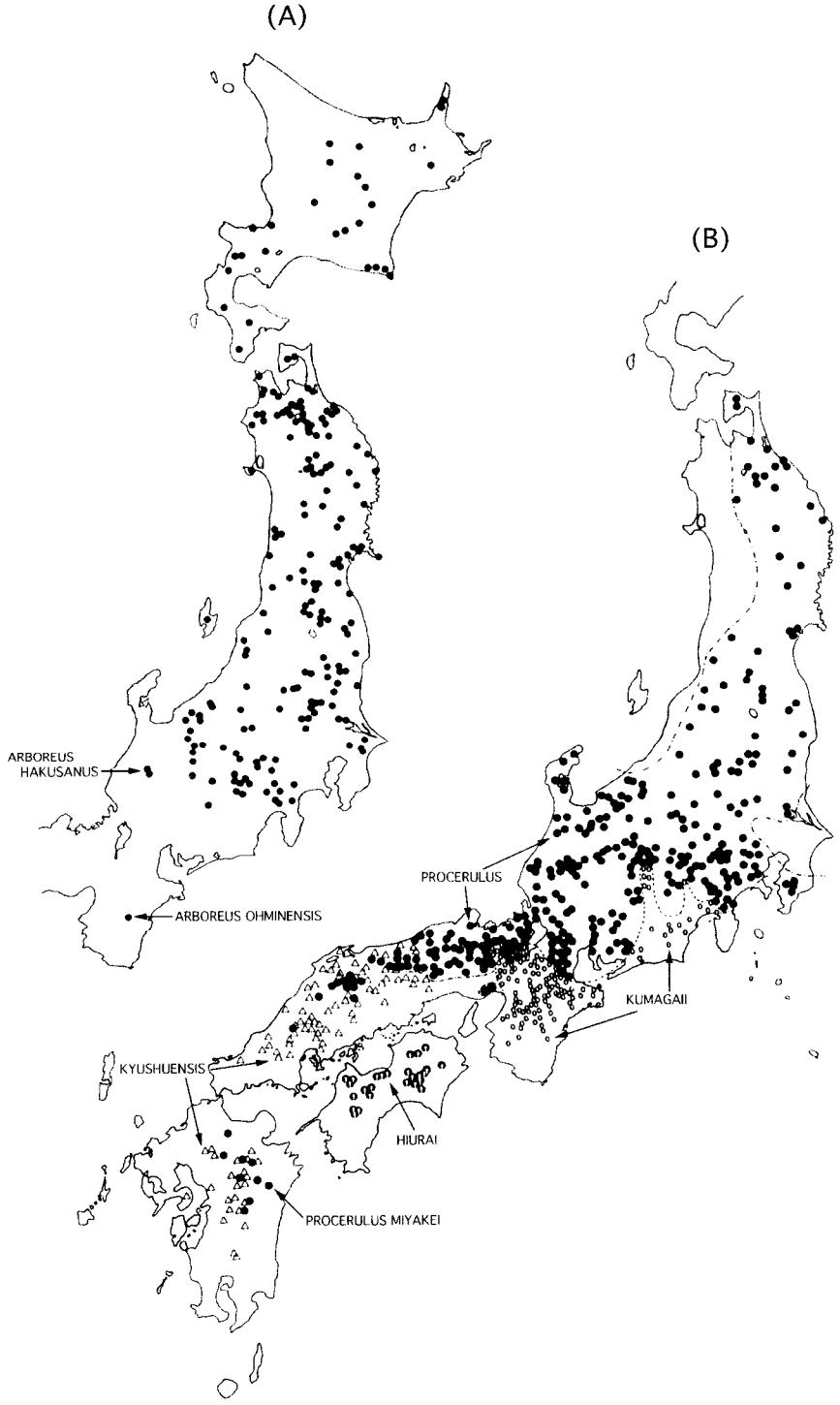

Fig. 1. Distribution ranges of *Leptocarabus arboreus* (A) and other *Leptocarabus* species (B) in the Japanese Islands (after Tominaga and Hiura, l979, slightly modified). Dots and other symbols denote the localities of specimens for identification by morphological characters and not those for the DNA analyses.

L. (*Aulonocarabus*) *kurilensis* is excluded from this study, because of no direct phylogenetic relation of this species to the other Japanese *Leptocarabus* species (Kim et al., 2000; Tominaga et al., 2000). *Tomocarabus opaculus* and *T. harmandi* are sometimes treated as belonging to *Leptocarabus* but are not dealt with in this paper, because each of them constitutes a lineage independent from *Leptocarabus* (Su et al., 2000).

MATERIALS AND METHODS

The specimens used in this study are listed in Table 1. Identifications of the specimens were done by O. T., one of the authors of the present paper. The specimens and the DNA samples thereof are preserved in the JT Biohistory Research Hall. The methods employed are the same as described previously for the ND5 gene sequence analyses and construction of the phylogenetic trees by the unweighted pair-group method (UPGMA) and the neigh-

Table 1. List of specimens analysed

Morphological species	No. of individuals	Locality	DDBJ/EMBL/GenBank Accession No. (ND5)
Leptocarabus arboreus			
	1	Naruseppu, Hokkaido	AB047404
	2	Ikutahara, Hokkaido	AB047405
	3	Kitami, Hokkaido *	AB031463
	4	Kushiro, Hokkaido	AB047406
	5	Nemuro, Hokkaido	AB047407
	6	Taiki, Hokkaido	AB047408
	7	Shizunai, Hokkaido	AB047409
	8	Niikappu, Hokkaido	AB047410
	9	Atsuma, Hokkaido	AB047411
	10	Oshamanbe, Hokkaido	AB047412
	11	Fukushima, Hokkaido *	AB031462
	12	Hakodate, Hokkaido *	AB031461
	13	Mt. Chokaizan, Akita Pref.	AB047413
	14	Kushibiki, Yamagata Pref.	AB047414
	15	Otama, Fukushima Pref.	AB047415
	16	Tanagura, Fukushima Pref.	AB047416
	17	Yaita, Tochigi Pref. *	D50355
	18	Nikko, Tochigi Pref.	AB047417
	19	Tsumagoi, Gunma Pref.	AB047418
	20	Mutsu, Aomori Pref. *	AB031467
	21	Mt. Osorezan1, Aomori Pref.	AB047419
	22	Mt. Osorezan, Aomori Pref.	AB047420
	23	Yokohama, Aomori Pref.	AB047421
	24	Nobeji, Aomori Pref.	AB047422
	25	Iwasaki, Aomori Pref.	AB047423
	26	Minehama1, Akita Pref.	AB047424
	27	Minehama2 Akita Pref.	AB047425
	28	Tashiro, Akita Pref.	AB047426
	29	Owani, Aomori Pref.	AB047427
	30	Takko, Aomori Pref.	AB047428
	31	Shingo1, Aomori Pref.	AB047429
	32	Shingo2, Aomori Pref.	AB047430
	33	Kuraishi2, Aomori Pref.	AB047431
	34	Morioka, Iwate Pref. *	AB031464
	35	Hiraizumi, Iwate Pref.	AB047432
	36	Kurikoma, Miyagi Pref. *	AB031465
	37	Shirataka, Yamagata Pref.	AB047433
	38	Sanpoku, Niigata Pref.	AB047434
	39	Shibata, Niigata Pref.	AB047435
	40	Nishiaizu, Fukushima Pref.	AB047436
	41	Chino, Nagano Pref. *	AB031468
	42	Fujimi, Nagano Pref.	AB047437
	43	Yamanaka-ko, Yamanashi Pref.	AB031466
	44	Mt. Hakusan, Ishikawa Pref.	AB047438
	45	Mt, Ohmine, Nara Pref.	AB047439
L. procerulus			
	46	Mutsu, Aomori Pref. *	AB031441
	47	Mt. Osorezan2, Aomori Pref.	AB047441
	48	Kuraishi1, Aomori Pref.	AB047442
	49	Hachinohe, Aomori Pref.	AB047443
	50	Ugo, Akita Pref.	AB047444
	51	Hiraizumi, Iwate Pref.	AB047445
	52	Murayama, Yamagata Pref. *	AB031445
	53	Murakami, Nigata Pref. *	AB031447
	54	Yaita, Tochigi Pref.	AB047446
	55	Shinano, Nagano Pref.	AB047447
	56	Togakushi1, Nagano Pref.	AB047448
	57	Togakushi2, Nagano Pref.	AB047449
	58	Karuizawa, Nagano Pref.	AB047450
	59	Fujimi, Nagano Pref.	AB047451
	60	Gonbe-toge, Nagano Pref. *	D50357
	61	Ashiyasu, Yamanashi Pref.	AB047452
	62	Fujinomiya, Shizuoka Pref.	AB047453
	63	Unazuki, Toyama Pref.	AB047454
	64	Wajima, Ishikawa Pref.	AB047455
	65	Maze, Gifu Pref. *	AB031442
	66	Mt. Hakusan, Ishikawa Pref.	AB047456
	67	Kiyomizu, Fukui Pref.	AB047457
	68	Shin-asahi, Shiga Pref.	AB047458
	69	Suzuka, Mie Pref. *	AB031444
	70	Asago, Hyogo Pref. *	AB031446
	71	Kuchiwa, Hiroshima Pref.	AB047459
	72	Mt. Hikosan, Fukuoka Pref.	AB031440
	73	Hinokage, Miyazaki Pref.	AB031439
L. kumagaii			
	74	Minobu, Yamanashi Pref. *	AB031450
	75	Ina, Nagano Pref.	AB047460
	76	Takayama, Gifu Pref. *	AB031448
	77	Hirakata, Osaka Fu *	AB031449
	78	Sakurai, Nara Pref.	AB047461
	79	Miyakawa, Mie Pref.	AB047462
	80	Omiya, Mie Pref.	AB047463
L. hiurai			
	81	Hojo, Ehime Pref.	AB047464
	82	Saijo, Ehime Pref. *	AB031436
	83	Hongawa, Kochi Pref. *	AB031437
	84	Higashi-iyayama, Tokushima Pref. *	AB031438
L. kyushuensis			
	85	Fukue, Yamaguchi Pref.	AB047465
	86	Mt. Unzendake1, Nagasaki Pref.	AB047466
	87	Kunimi, Nagasaki Pref.	AB047467
	88	Kunimi2, Nagasaki Pref.	AB047468
	89	Mt. Unzendake2, Nagasaki Pref.	AB047469
	90	Obama, Nagasaki Pref.	AB047470
	91	Kurayoshi, Tottri Pref. *	AB031431
	92	Matsue, Shimane Pref. *	AB031432
	93	Tojo, Hiroshima Pref.	AB047471
	94	Higashi-hiroshima, Hiroshima Pref.	AB047472
	95	Kure, Hiroshima Pref. *	D50356
	96	Mt. Kanmuriyama, Hiroshima Pref. *	AB031430
	97	Mt. Kinposan, Kumamoto Pref.	AB047473
	98	Otsu, Kumamoto Pref. *	AB031434
	99	Mt. Aso, Kumamoto Pref.	AB047474
	100	Gokase, Miyazaki Pref. *	AB031433
	101	Hitoyoshi, Kumamoto Pref. *	AB031435

* Taken from Su et al. 1996, and Kim et al. 2000.

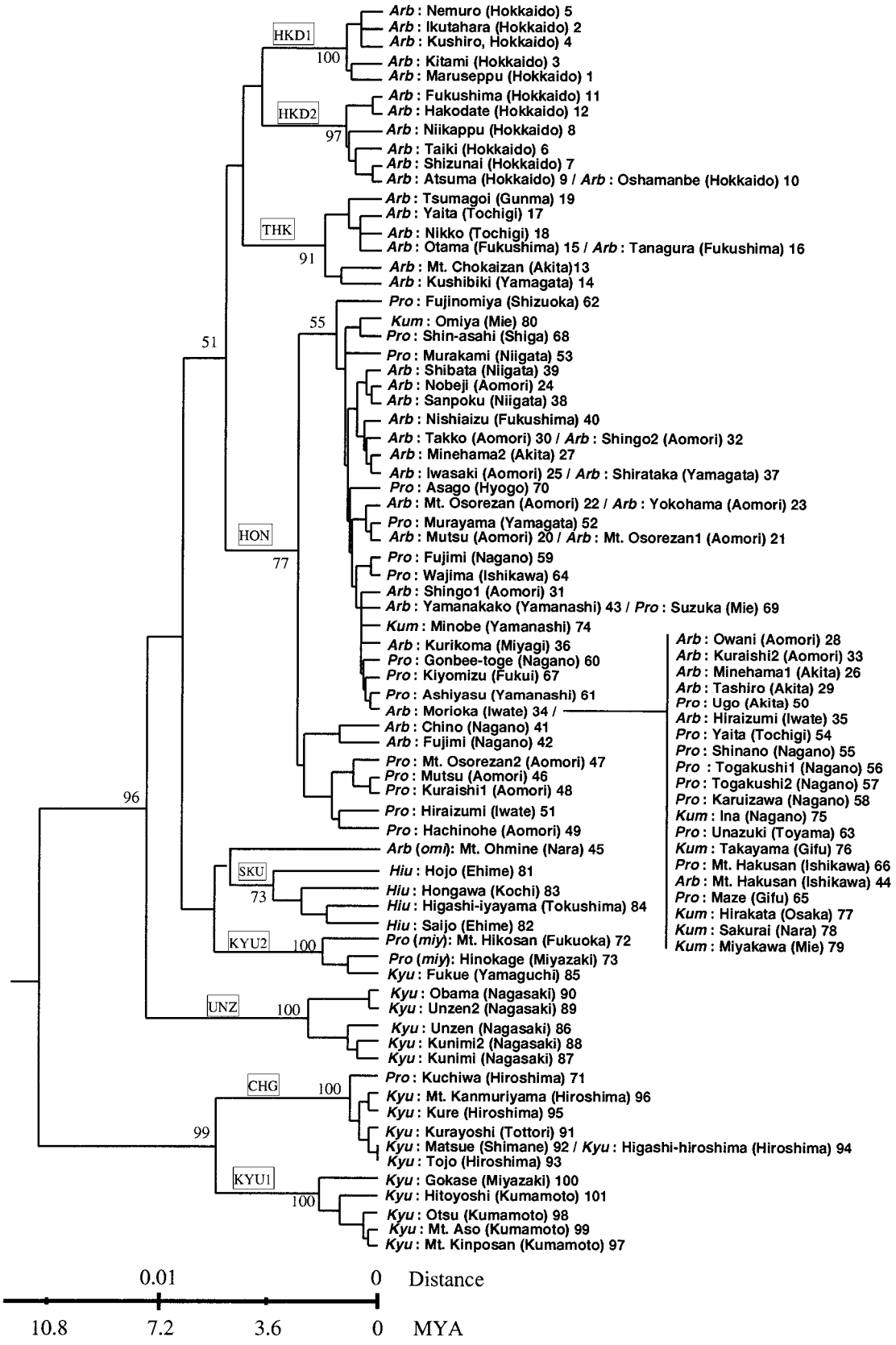

Fig. 2. Phylogenetic tree (UPGMA) of the mitochondrial ND5 gene of the Japanese *Leptocarabus* species. Distance (D) denotes Kimura's two-parameter evolutionary distance (Kimura, 1980). The value at the node represents bootstrap confidence level (%) based on 500 resamplings. Arb: *Leptocarabus arboreus*, Pro: *L. procerulus*, Kum: *L. kumagaii*, Hiu: *L. hiurai*, Kyu: *L. kyushuensis*.

bor-joining (NJ) method with bootstrap test (e.g., Su et al., l998; Kim et al., 2000). Supplementary analyses were made using mitochondrial cytochrome oxidase subunit I (COI) gene for which the 1,036 bp sequence of the COI gene was determined after amplification of the gene using the following two mixed primers: forward primer 5'-CGC TCT AGA ACT AGT GGA TCA CNA AYC AYA ARG AYA TYG GNA C-3' (SKCOI-7) and reverse primer: 5'-TCG AGG TCG ACG GTA TCA CRT ART GRA ART GNG CNA CNA CRT ART A-3' (KSCOI-2). Two nuclear genes, i.e., nuclear 28S rRNA (see Kim et al., 2000) and trehalase (1,004 bp) (Su et al., unpublished), were also examined (see Results).

RESULTS

Throughout the ND5 and COI gene sequences used, neither deletions or insertions were required for multiple alignment. Most substitutions were found at the codon silent sites. Multiple substitutions were corrected by Kimura's formula (Kimura, l980). The evolutionary distance (D) and chronology for several carabid beetles, which are phylogenetically remote from each other, are linearly correlated at least within a D range up to 0.07, suggesting the near-constancy of the rate of base substitution throughout various carabid groups (Su et al., l998; Osawa et al., l999). Thus, the UPGMA can be used to construct the phylogenetic tree. The NJ method gave essentially the same topology (not shown) as that by the UPGMA.

Fig. 2 shows a phylogenetic tree of the mitochondrial ND5 gene from 101 specimens of the *Leptocarabus* species collected at various localities that cover almost all the distribution ranges in the Japanese Islands. As shown in a previous paper (Kim et al., 2000), two major lineages of the mitochondrial haplotype were recognized. The separation of these two lineages was calculated to have taken place about 11 million years ago (MYA), assuming that a 0.01 D unit corresponds to 3.6 million years (MYR) according to Su et al. (l998, 2001; see also Osawa et al., 1999; Tominaga et al., 2000). The lineage 1 included *L. kyushuensis* (with one exception, see below), which was further divided into two sublineages. The first one (KYU1 in Fig. 2) contained *L. kyushuensis* from the mainland Kyushu and the second one (CHG) contained all the specimens of *L. kyushuensis* from the Chugoku district of Honshu, and one specimen identifiable as *L. procerulus* from Kuchiwa, Hiroshima Prefecture, Honshu. The separation of the sublineages 1 and 2 was estimated to have occurred about 5–7 MYA. The sequence diversification within each sublineage was rather small.

The lineage 2 was composed of at least 6 sublineages. The first one (UNZ) was represented solely by *L.*

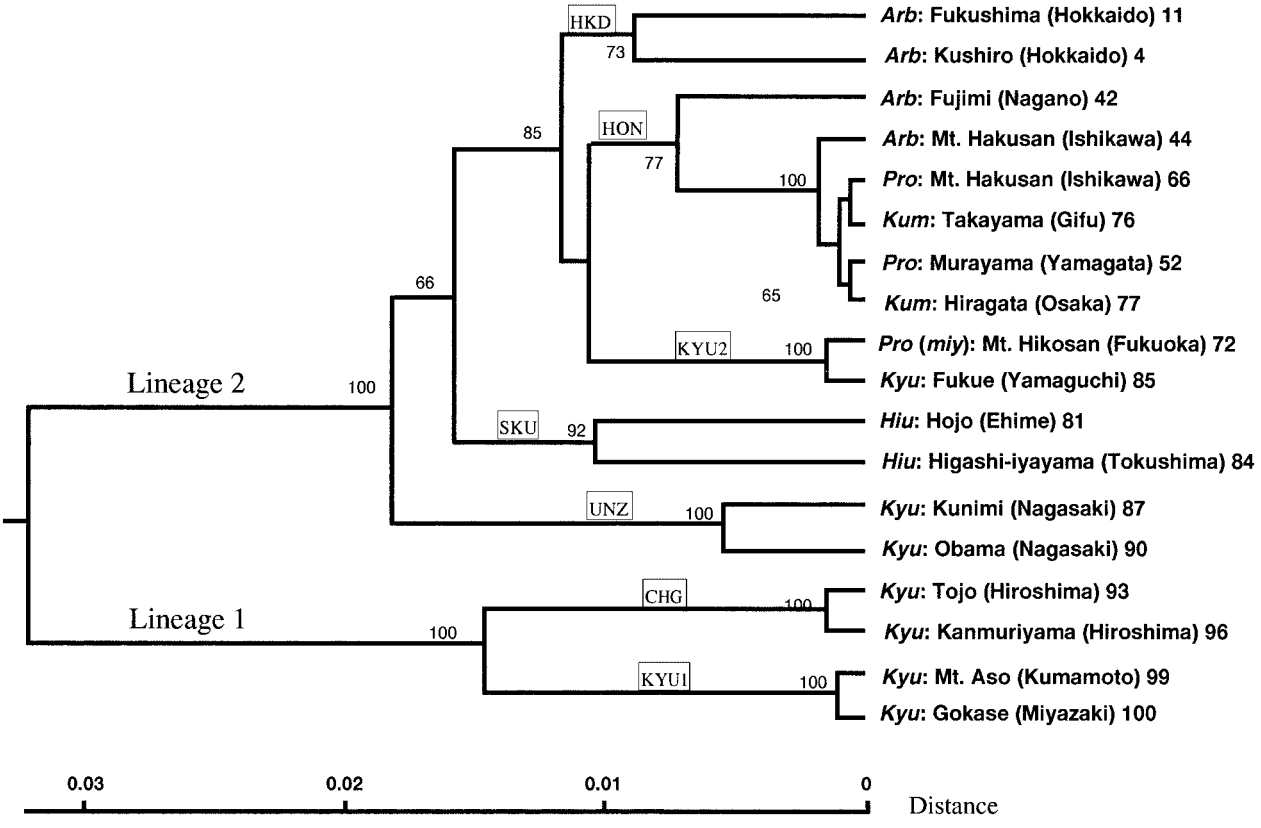

Fig. 3. Phylogenetic tree (UPGMA) of the mitochondrial COI gene of the representative Japanese *Leptocarabus* species. The sequence data will appear in the DDBJ/EMBL/GenBank databases with the accession numbers AB047562-AB047579.

kyushuensis from Shimabara Peninsula, Nagasaki Prefecture, Kyushu (Kim et al., to be published). The second one (KYU2) contained *L. procerulus miyakei* from Kyushu and one specimen from Fukue, Yamaguchi, Honshu belonged to this sublineage. The third sublineage (SKU) was composed of *L. hiurai* from Shikoku and unexpectedly *L. arboreus ohminensis* from Mt. Ohmine, Nara Prefecture, Honshu. Diversification of the ND5 sequences in this sublineage is rather old. The fourth one (HON) was composed of a mixture of three different species, *L. procerulus*, *L. kumagaii* and *L. arboreus* which are widely distributed from the Kinki district up to the northernmost Honshu. *L. procerulus* is distributed throughout the distribution range of HON, while *L. kumagaii* occupies the Kinki and Chubu districts, and *L. arboreus* inhabits mostly northern half of the HON range as has been known from the taxonomic studies. The sequence divergence was very small among all the specimens in this sublineage, and the sequence of many of them were identical. The mitochondrial haplotypes of the three species were intermingled on the trees and there were no species-specific clusters throughout. The fifth sublineage THK consisted of only *L. arboreus*, the distribution range of which was embedded within HON in the Tohoku district. The sixth sublineage HKD comprised *L. arboreus* from Hokkaido and was further divided into HKD1 and HKD2 which were roughly bordered by the Hidaka mountains.

A phylogenetic tree was also constructed using the COI sequences for the representative species of the respective sublineages (Fig. 3). Both the ND5 and COI trees yielded the same topology for the species examined. The nuclear trehalase gene and 28S rDNA were also sequenced. However, the sequence divergence of the nuclear genes from the Japanese *Leptocarabus* species was so small (1/5 to l/10 of the ND5 gene) that no meaningful phylogenetic trees could be constructed.

DISCUSSION

Intermingled occurrence of more than two mitochondrial DNA haplotypes in a single phylogenetic line There are a number of cases in which different species appear in the same mitochondrial haplotype lineage. In the CHG sublineage of the lineage 1, *L. procerulus* sympatrically occurs among *L. kyushuensis*. In the KYU2 sublineage of the lineage 2, *L. kyushuensis* appears allopatrically with *L. procerulus*. *L. arboreus ohminensis* from Mt. Ohmine, Nara belongs to the same SKU sublineage of the lineage 2 with *L. hiurai*. These two are distributed allopatrically. An extreme of such an intermingled occurrence of more than two species is seen in the sublineage HON of the lineage 2, where *L. procerulus*, *L. kumagaii* and *L. arboreus* appear either sympatrically (e.g., *L. procerulus* and *L. arboreus hakusanus* at Mt. Hakusan, Gifu, Honshu; locality nos. 66 and 44 in Fig. 2 and Table 1) or allopatrically (e.g., *L. procerulus* and *L. kumagaii* in the Kinki district, etc). These Japanese *Leptocarabus* species are taxonomically separated by external morphology and genital organs. In particular, morphology of male genital organ is the key to distinguish the species. Based on the morphological characters, *L. arboreus* is often separated from the other *Leptocarabus* species as belonging to the distinct subgenus, *Adelocarabus*. How can this phenomena be interpreted ? One explanation would be that the similar morphologies in different lineages in the mitochondrial DNA might result from hybridization between two or more species. It might be the case for the sympatrically or parapatrically distributed species, but it is hard to imagine the occurrence of hybridization between two allopatrically distributed species such as *L. hiurai* in Shikoku and *L. arboreus ohminensis* from Mt. Ohmine, Honshu. These two are separated by the Kitan Strait and *L. arboreus ohminensis* is sharply isolated geographically from the other *L. arboreus* subspecies, the distribution range of which is far distant from that of *L. a. ohminensis*. In the sublineage HON in the lineage 2, the haplotype DNA sequences in many specimens of *L. procerulus*, *L. kumagaii* and *L. arboreus*, especially those inhabiting the Kinki, the Chubu and a part of the Tohoku district, are identical or very close, and no species-specific DNA sequences have been found (Fig. 2). These facts are not in accord with, although not completely exclude, the hybridization hypothesis. Very little divergence of the mitochondrial haplotype sequences mentioned above suggests a recent emergence of at least two species out of three. An alternative explanation would be that two or more "morphological" species of *Leptocarabus* such as *L. procerulus* and *L. arboreus* or else merely represent the morphological polymorphism within the same species belonging to the same reproductive population and this is worthwhile considering. However, as there is no evidence for this, one has to follow the traditional classification system proposed by the majority taxonomists in this field.

Still another possibility, which we have previously proposed (Su et al., 1996; Kim et al., 2000), would be that discontinuous transformation from one type (species) to another took place in different phylogenetic lines in various periods in evolution. Most of the examples of the intermingled occurrence of more than two morphological species of *Leptocarabus* in the same lineage may be explained by this hypothesis. Such a probable morphological transformation, which we call "type switching", has been observed in a number of carabid lines (Su et al., l996; Imura et al., l997; Kim et al., l999; Kim et al., 2000; for review, see Osawa et al., l999). Taken altogether, we suggest that morphological differentiation is in many cases discontinuous, and does not always run parallel with phylogeny.

Formation of the Japanese *Leptocarabus* fauna From the phylogenetic tree (Fig. 2) and the distribution map of the lineages and sublineages (Fig. 4), it is inferred that the ancestry of all the Japanese *Leptocarabus* species had inhabited the ancient Japan area (presumably somewhere in the ancient northern Kyushu area), which had the morphology of the *L. kyushuensis*-type, and separated into two lineages, the lineage 1 and the lineage 2, about 11 MYA after split of the Japanese Islands from the Eurasian Continent ca. 15 MYA (Su et al., 1998; Tominaga et al., 2000). The lineage 1 then split into two sublineages KYUl and CHG, and the latter invaded the Chugoku district, Honshu, followed by geographic isolation from KYU1. In both of these two sublineages, the *L. kyushuensis*-type morphology has been maintained except some specimens in Hiroshima Prefecture, Honshu, that can be identified as *L. procerulus*. In the lineage 2, the sublineage UNZ separated from the rest of the sublineages

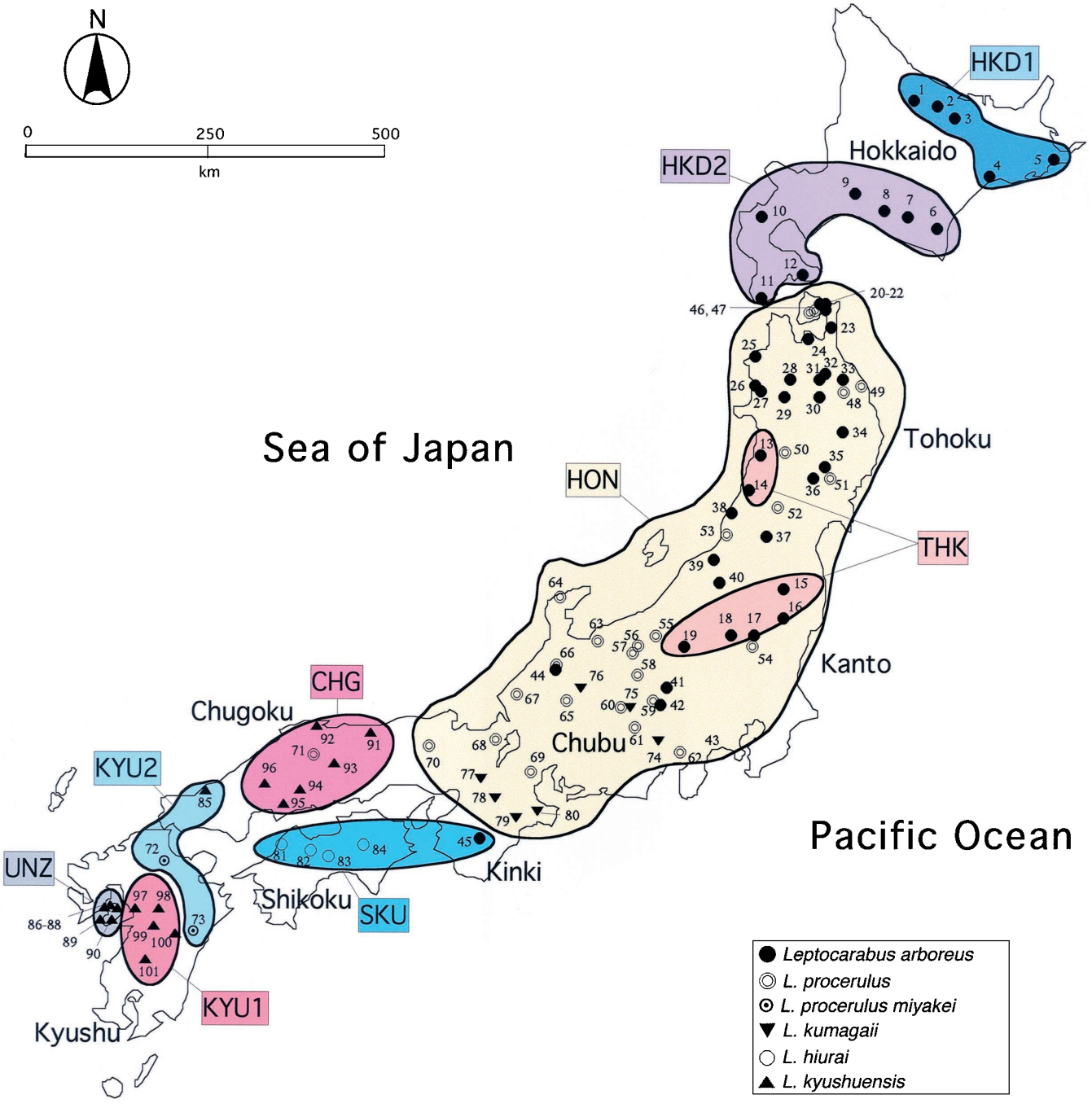

Fig. 4. Distribution map of the lineages and sublineages of the Japanese *Leptocarabus* species used in this study. Locality numbers correspond to those shown in Table 1, and Figs. 2 and 3.

and isolated around the Shimabara Peninsula (Kim et al., to be published), having kept the *L. kyushuensis*-type morphology. From the other branch of the lineage 2, five sublineages emerged. Since these have either the *L. procerulus*- or the *L. arboreus*-type morphology, a morphological transformation is assumed to have occurred after split from the UNZ sublineage. The ancestor of these sublineages rapidly propagated its distribution to northeast and southeast, forming the respective sublineages in different areas in the Japanese Islands presumably as a result of geographic and/or ecological isolations, followed by expansion or restriction of their distribution ranges. These sublineages are: (1) KYU2 consisting of *L. procerulus miyakei* in northern Kyushu with one specimen from Yamaguchi, Honshu that has however the *L. kyushuensis*-type morphology, (2) SKU in Shikoku (*L. hiurai*) and Mt. Ohmine, Nara, Honshu (*L. arboreus ohminensis*). (KYU2 and SKU have some affinity on the trees. Because of a low bootstrap value between KYU2 and SKU, these two sublineages may not be sharply separable.) (3) HON from the Kinki district to the northern tip of Honshu, containing *L. procerulus*, *L. kumagaii* and *L. arboreus*, (4) THK in the restricted area of the Tohoku district (*L. arboreus*), and (5) HKD in Hokkaido (*L. arboreus*). The ancestor of these five sublineages would have had the *L. procerulus*-type morphology, because *L. procerulus* and its congeners inhabit most widely throughout the distribution range of the lineage 2. Note that *L. hiurai* was first described as a subspecies of *L. procerulus*, and *L. kumagaii* was not distinguished from *L. procerulus*. Perhaps, during expansion of the distribution range of each sublineages, morphological transformation would have often occurred as mentioned in the previous section. *L. arboreus* seems to be adapted to the alpine/subalpine environments in northeastern Japan, while *L. procerulus or L. kumagaii* seems to prefer the moderate habitats mainly in central Japan and the Kinki district, although *L. procerulus* inhabits the area where *L. arboreus* lives. Transformation from the *L. procerulus*-type morphology to *L. arboreus* might have taken place in the northeastern areas by the above ecological reasons.

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